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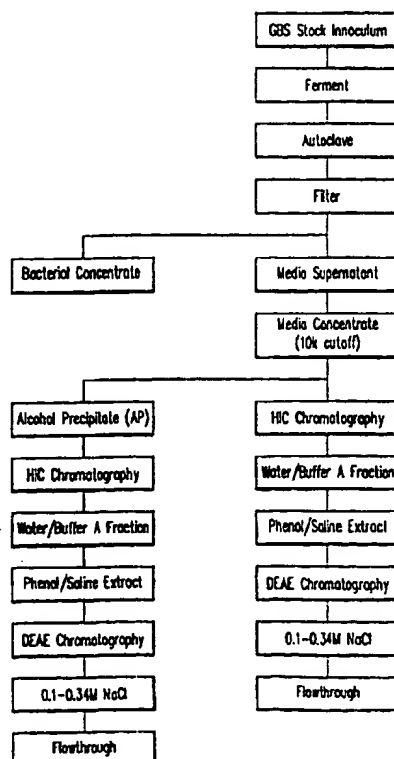
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(54) Title: METHOD FOR PURIFYING GBS TOXIN/CM101

(57) Abstract

A method for purifying a polysaccharide from group B β -hemolytic *Streptococcus* (GBS) bacteria includes contacting a bacterial fermentation stock with a hydrophobic interaction chromatography (HIC) resin. Additional steps may include a phenol/saline extraction and an ion exchange chromatography. The method results in a product having very high purity. The product of the purification provides a composition which is highly useful in both research and therapeutic settings.



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METHOD FOR PURIFYING GBS TOXIN/CM101

INTRODUCTION

Technical Field

This invention relates to improved methods of purification for a polysaccharide.

Background

CM101, a GBS toxin, is a pathogenic molecule isolated from group B β -hemolytic *Streptococcus* (GBS) bacteria. Newborn infants may become infected with GBS, a condition known as GBS pneumonia or "early-onset disease," and suffer from sepsis, granulocytopenia, and respiratory distress, i.e. pulmonary hypertension and proteinaceous pulmonary edema (Hellerqvist, C.G. et al., *Studies on group B β -hemolytic streptococcus I. Isolation and partial characterization of an extra-cellular toxin.*, Pediatr. Res., 12:892-898 (1981)).

Despite the harmful effects to neonates exposed to GBS, CM101 is not known to cause toxicity in older humans. In fact, research into this toxin has revealed a significant therapeutic application. See U.S. Patent No. 5,010,062 and Hellerqvist, C.G. et al., *Early Results of a Phase I Trial of CM101 in Cancer Patients.*, Proceedings of the American Association of Cancer Research Annual Meeting (1995), wherein CM101 is utilized to inhibit vascularization of tumors. Obtaining purified CM101 is critical, therefore, for both research and therapeutic purposes.

CM101 is a complex polysaccharide toxin having a molecular weight of approximately 300,000 Daltons and comprising N-acetyl-galactosamine, N-acetyl-glucosamine, glucose, galactose, and mannose residues. Nmr (nuclear magnetic resonance) results suggest that alditol residues may also be present. Carboxylic acid functional groups, probably galacturonic acid, are also believed to be an integral part of the molecule. Repeating active epitopes most likely play an important role in the pathophysiological response to CM101 by crosslinking

1 receptors on target endothelium (Hellerqvist, C.G. et al., *Early Results of a Phase*
2 *I Trial of CM101 in Cancer Patients.*, Proceedings of the American Association of
3 Cancer Research Annual Meeting (1995); DeVore, R.F., et al., *A Phase I Study of*
4 *the Antineovascularization Drug CM101*, J. Clin. Can. Res., 3:365-372 (1997)).

5 U.S. Patent No. 5,010,062 provides a method of purification of a GBS
6 toxin. The method taught is labor-intensive, however, requiring numerous steps
7 with continual levels of loss of biological activity.

8 Purification of CM101 as presently known in the art provides an end
9 material which is only 40% pure as measured by chemical analyses and biological
10 assays. The other 60% comprises plant and yeast polysaccharides and endogenous
11 bacterial polysaccharides. The plant and yeast contaminants originate for the most
12 part in the additives to the commercial culture media used for optimal growth of
13 the GBS bacteria. The endogenous contaminants include GBS polysaccharides
14 including group and type specific antigens (Paoletti, L.C. et al., *Neonatal mouse*
15 *protection against infection with multiple group B streptococcal (GBS) serotypes by*
16 *maternal immunization with a tetravalent GBS polysaccharide-tetanus toxoid*
17 *conjugate vaccine*, Infect. Immun. 62(8):3236-43 (1994); Michon, F.,
18 *Multiantennary group-specific polysaccharide of Group B Streptococcus*,
19 Biochem., 27:5341-51 (1988)). CM101 of this 40% purity level represents the
20 current clinical grade. There is a need, therefore, for a purification method of
21 CM101 which results in an end product with increased overall purity, preferably
22 with the removal of extraneous plant and yeast polysaccharides and GBS antigenic
23 polysaccharides.

24 Additionally, the purification scheme known in the art includes
25 environmentally unsound steps, such as the use of a large volume of phenol in a
26 phenol:water extraction. Phenol is a well-known caustic material.

27 Therefore, objects of the present invention are to provide a purification
28 method resulting in (i) a material of high purity, (ii) using a minimal number of
29 steps, (iii) minimizing the use of caustic or toxic materials such as phenol, and (iv)
30 increasing the yield of material.

31
32

SUMMARY OF THE INVENTION

The above objects have been achieved with the invention described herein. Particularly, a purification scheme including a hydrophobic interaction chromatography (HIC) resin for purification of CM101 from GBS bacterial culture media results in a product of greater than 95 % purity.

One aspect of this invention is a process for purifying a polysaccharide toxin from GBS bacteria, the process including the use of an HIC resin. The present invention also includes a substantially pure polysaccharide toxin from GBS bacteria produced by the method disclosed herein, and a pharmaceutical composition comprising a substantially pure toxin and a pharmaceutically acceptable carrier. The pharmaceutical composition may be used to treat a patient having a medical condition. For example, a tumor patient may be treated with the composition of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a CM101 purification scheme of the present invention.

Fig. 2 illustrates a known CM101 purification scheme.

Fig. 3 is a quantitative hydrolysis standard curve showing the dose response of a PAD detector for 5, 20, and 50 μ g of dextran (a glucose polymer) with 6-deoxy glucose as a constant internal standard.

Fig. 4 shows the separation of standard sugar samples.

Figs. 5a-b are elution profiles of a media concentrate on a butyl-Sepharose HIC column. Fig. 5a is measured at UV 206 absorbance. Fig. 5b is measured at UV 280 absorbance.

1 **Fig. 6a** is an HPLC profile of an HIC-purified water-eluted fraction containing
2 CM101 (16min peak) and monitored at UV 203 absorbance on a Millennium 2000
3 Diodo-Ray detector (Waters, Millford, MA).

4
5 **Fig. 6b** is a Diodo-Ray spectrum corresponding to **Fig. 6a** and illustrating minimal
6 presence of 260 absorption (RNA and DNA) and 280 absorption (tyrosine-
7 containing protein) for the CM101 containing (16 min) peak.

8
9 **Fig. 7a** is an elution profile monitored at 203nm showing the purity of the HIC
10 water-eluted peak of **Fig. 6a** further subjected to phenol/saline extraction and
11 subsequent DEAE chromatography.

12
13 **Fig. 7b** is a Diodo-Ray spectrum illustrating the purity of the CM101-containing
14 peak of **Fig. 7a** as evidenced by the narrow symmetric peak and the lack of
15 absorption at 260nm (RNA/DNA) and 280nm (protein).

16
17 **Fig. 8** is a profile of IL-6 activity by ANA-1 Assay of fractions obtained from an
18 HIC column, more specifically an IL-6 activity profile of fractions obtained from
19 10K5P6 concentrate run on 100ml Butyl Sepharose (FT = flow-through; 1M =
20 1M phosphate fraction; 0.25M = 0.25M phosphate fraction; H₂O = water
21 fraction; EtOH = ethanol fraction).

22
23 **Fig. 9** illustrates a sugar analysis of CM101 purified by the method of the present
24 invention.

25
26 **Fig. 10** is an HPLC profile of current clinical grade CM101 further subjected to
27 HIC chromatography.

28
29 **Fig. 11** illustrates a sugar analysis of a sample of current clinical grade CM101
30 which was further purified by HIC and HPLC.

31

1 **Fig. 12a** is an HPLC profile of CM101 purified by a known process using 10mM
2 phosphate buffer, pH 8.4.

3
4 **Fig. 12b** is an HPLC profile of CM101 purified by the method of the invention,
5 using the same running conditions as the HPLC profile of **Fig. 12a**.

6
7 DESCRIPTION OF SPECIFIC EMBODIMENTS

8
9 GBS toxin as used herein is defined as any fraction or component isolated
10 from natural or lysed GBS bacteria, or derived from media supernatants of lysed
11 and/or autoclaved GBS bacteria, and which has a biological activity evidenced by
12 induction of respiratory distress in the sheep assay (Hellerqvist, C.G. et al.,
13 *Studies on group B β -hemolytic streptococcus I. Isolation and partial*
14 *characterization of an extra-cellular toxin.*, Pediatr. Res., 12:892-898 (1981)) or
15 activation of complement and binding to neovasculature as demonstrated by a
16 peroxidase-antiperoxidase (PAP) assay of a tumor tissue specimen (Hellerqvist,
17 C.G. et al., *Anti-tumor effects of GBS toxin: a polysaccharide exotoxin from group*
18 *B β -hemolytic streptococcus*, J. Canc Res. Clin. Oncol., 120:63-70 (1993); and
19 Hellerqvist, C.G. et al., *Early Results of a Phase I Trial of CM101 in Cancer*
20 *Patients.*, Proceedings of the American Association of Cancer Research Annual
21 Meeting (1995)).

22 Substantially pure GBS toxin means a preparation in which GBS toxin is
23 greater than 40% pure (e.g., present in a concentration of at least about 40% by
24 weight), preferably at least approximately 60% pure, more preferably at least
25 approximately 90% pure, and most preferably at least approximately 95% pure.

26 A source for GBS starting material for use in the method of the present
27 invention may be obtained by culturing strains of Group B *β -hemolytic*
28 *Streptococcus* bacteria that have recently infected or are capable of infecting
29 newborn infants. Isolates of such strains may be obtained from the blood of
30 infected infants.

31 High production of CM101 generally requires fermentation with the
32 complex media THB which contains high molecular weight material in the form of

1 polysaccharides and proteins for GBS optimum growth and CM101 production.
2 During the fermentation process, the bacteria produce from the nutrients quantities
3 of proteins, nucleic acids, and polysaccharides other than CM101. The estimated
4 concentration of CM101 in the fermentation broth is less than 0.1 % by weight.

5 The purification method of the present invention employs hydrophobic
6 interaction chromatography (HIC) which eliminates the bulk of the endogenous and
7 exogenous contaminating proteins, nucleic acids, and polysaccharides more
8 efficiently than known methods and results in an end product which contains 10-
9 50% pure CM101. In just one step of contacting the GBS starting material and
10 the HIC resin, this represents a 100-500 fold purification from the starting
11 material.

12 Use of an HIC resin for purification of a polysaccharide is surprising and
13 novel because HIC columns are designed for purification of hydrophobic proteins
14 and are not believed useful for polysaccharides free of proteins and lipids.
15 Polysaccharides are generally characterized as being hydrophilic due to their
16 numerous hydroxyl groups. Application of a starting material to an HIC column
17 under the conditions recommended by the manufacturer and used by practitioners
18 skilled in the art would therefore be with the intention of retaining proteins and
19 allowing polysaccharides to pass through the column unbound.

20 The surprising discovery is that CM101 has hydrophobic properties that
21 allow use of the present purification scheme to achieve a high level of purity.
22 Especially surprising is that CM101 has significantly more hydrophobic
23 characteristics than most of the proteins and polysaccharides present in the
24 supernatant from which the CM101 is isolated. Greater than 98% of these protein
25 and polysaccharide contaminants pass through the HIC column. Although the HIC
26 resin is generally employed in an HIC column, this step alternatively may be
27 performed by contacting the resin and the starting material in some other manner.
28 For example, the GBS source and the resin may be placed in a vessel together in a
29 batchwise process, and the toxin-containing portion subsequently separated from
30 the resin as by centrifugation.

31 Additional purification steps may include a phenol/saline extraction in a
32 small volume relative to the prior methods (approximately 1000-fold reduced) and

1 an ion exchange column. These additional purification steps contribute to an end
2 product with greater than 95% purity.

3 HIC is a method used to separate proteins, such as membrane proteins,
4 based on their hydrophobic nature. An HIC resin is defined as a resin having
5 interactive hydrophobic groups which are generally covalently attached to a
6 support such that the hydrophobic groups are free to interact with substances in
7 contact with the resin. Examples of hydrophobic groups include alkyl, alkoxy,
8 and aryl groups. The preferred HIC resin to be used in accordance with the
9 present invention has a support with attached aliphatic groups of two or more
10 carbons, preferably alkyl groups in the range of 2 to 12 carbons, and more
11 preferably normal or branched butyl groups. Phenyl groups or alkoxy groups of
12 up to 20 carbons are also preferred interactive hydrophobic groups. The
13 interactive hydrophobic groups are preferably supported by Sepharose
14 (Pharmacia), acrylamide (Toso Haas, Montgomeryville, PA), or silica. According
15 to the standard procedure for use of an HIC column, the starting material
16 containing the protein of interest is applied to the column in up to 2M aqueous salt
17 solution and the bound proteins are then eluted and separated through decreases in
18 hydrophobic interactions by reducing the ionic strength of the developing buffer.
19 Changes in pH and/or temperature may also be used to alter the hydrophobic
20 interactions.

21 CM101 purification from Group B *Streptococcus* requires obtaining a
22 bacterial culture of GBS. Bacterial inocula are incubated to late log phase in Todd
23 Hewitt Broth (THB) modified by supplementation with 2g/l or more of glucose
24 and Na₂HPO₄. As indicated in Fig. 1, the culture is then autoclaved. CM101 is
25 present in the supernatant of GBS fermentation cultures at a concentration of 2-15
26 mg/l following autoclaving. The media contains approximately 15 g/l of other
27 bacterial and media components. Thus, CM101 constitutes approximately 0.01-
28 0.1% of the components in the supernatant. After autoclaving, the media is
29 filtered. The filtrate is preferably concentrated via a 10,000 Dalton (10kD) cutoff
30 filter, although a filter having a cutoff of 50,000 Dalton (50kD) or less may be
31 used.

1 CM101 is then purified in accordance with this invention, as shown in Fig.
2 1, by applying a supernatant concentrate or reconstituted alcohol precipitate
3 thereof made 0.75-2M in potassium phosphate or another salt, such as sodium
4 phosphate, sodium or potassium sulphate, chloride, or acetate, to an HIC column
5 preferably equilibrated in the same salt of the same molarity. The method wherein
6 the media concentrate, or 10kD-50kD, starting material is employed without
7 alcohol precipitation and reconstitution is preferred because the media concentrate
8 starting material provides higher yield of CM101 than does the reconstituted
9 alcohol precipitate.

10 The CM101-containing starting material is applied to the HIC column and
11 washed with aqueous phosphate at 0.75-2M. Following a 0.75-2M wash, the
12 column is further developed with 0.5-1M and then 0.25M salt, preferably
13 phosphate. In the preferred embodiment, the CM101 is eluted from the column
14 with water as a single peak containing 10-50% CM101. Alternatively, water is
15 replaced for CM101 elution from the HIC column with 10mM phosphate, pH 6.8
16 in 10% ethanol in water (Buffer A), followed by 20% ethanol in water. CM101
17 activity is recovered in both the Buffer A and 20% ethanol fractions. Use of
18 Buffer A is generally not sufficient to remove all the CM101 from the HIC
19 column, so the Buffer A wash is followed by an additional 20% ethanol wash.
20 However, in scale-up, the ethanol constitutes an environmental hazard and the
21 subsequent phenol/saline extraction of the water peak or the Buffer A and 20%
22 ethanol peak fractions yields CM101 of approximately equal purity. The HIC
23 procedure removes better than 98% of both the proteins and media polysaccharides
24 remaining in the 10k concentrate or the reconstituted alcohol precipitate.

25 The enriched CM101 from the HIC column may be further purified by an
26 extraction in phenol and an aqueous salt solution, preferably 0.05M saline. This
27 additional step provides a CM101 fraction of approximately 95% purity.

28 The water or the combined Buffer A and 20% ethanol fractions eluted from
29 the HIC column are either dialyzed against water and lyophilized and reconstituted
30 in 0.05M saline or dialyzed against saline after concentration. Typically, phenol
31 is added to the material and the solution is rapidly heated to 70-80°C. When a
32 single phase forms, the solution is chilled to 4°C. The resulting saline phase of

1 the phenol/saline extraction contains CM101 and may then be applied to a cation
2 exchange column, such as DEAE.

3 For the DEAE column procedure, the DEAE column is equilibrated in
4 water and then washed with 0.1M saline, 0.05M NaOAc, pH 7.4 and developed
5 with a step gradient to 0.34M NaCl. Elution of CM101 is monitored and
6 quantitated with an ANA-1 assay. As with the HIC resin step, an ion exchange
7 resin may be contacted with the toxin-containing material through use of
8 equipment other than a column. The CM101-containing fraction is then dialyzed
9 against water and lyophilized. After the phenol/saline extraction and ion exchange
10 steps, CM101 is greater than 95% pure.

11 The column eluates, or material resulting from the resin contact steps, are
12 assayed for biologic activity with the ANA-1 and/or Dot Blot assay. The
13 biological activity is then confirmed with a sheep assay. Table 1 depicts several
14 separations of AP and 10k material obtained from different batches of starting
15 materials and applied to the HIC column. Removal of denatured protein and
16 media polysaccharides and other material is similar.

17 Although the preferred order of purification is to perform the HIC step,
18 followed by the phenol/saline extraction, and then the ion exchange step,
19 purification may also be performed in another order.

20 Fig. 2 presents an example of a known method of CM101 purification.
21 Notably, a 70% ethanol precipitate step is used, followed soon thereafter with a
22 phenol/water extraction. The large volumes of ethanol and phenol required at
23 these early stages of the known purification method represent environmentally
24 unsound practices. The method represented in Fig. 2 also requires an ion
25 exchange column, a gel filtration column, and a lentil lectin column.

26 The prior method contains numerous steps, including environmentally
27 hazardous ones. On the other hand, the method of the present invention is
28 effective, gives higher purity and 2 to 25 times the yield, and minimizes use of
29 environmentally unsound materials.

30 The environmentally hazardous phenol-water extraction step is reduced
31 1000-fold as compared to the previously used procedures. Furthermore, additional
32 purification as by a gel filtration procedure is eliminated. The lentil lectin

1 chromatography step of the prior method is also deleted. The end product of the
2 HIC column, phenol/saline extraction, and ion exchange column steps has
3 approximately 95% purity, so other treatments are unnecessary.

4 The CM101 purified by the method of the present invention may be used
5 for research or therapeutic purposes. The CM101 is particularly useful when
6 combined with a pharmaceutically acceptable carrier, e.g., reconstituted in saline
7 and administered to a patient intravenously. Other dosage forms to administer
8 purified CM101 may also be used. The pharmaceutical composition of this
9 invention comprises the substantially pure GBS toxin of this invention in
10 combination with a pharmaceutically acceptable carrier. In general, the carrier
11 will be one that is readily mixed with the toxin to form a composition that is
12 administrable by intravenous (IV) means. Thus, the carrier is preferably water,
13 which may have other pharmaceutically acceptable excipients included to ensure its
14 suitability for intravenous administration. The resulting composition will be sterile
15 and will have acceptable osmotic properties. In general, a suitable IV formulation
16 is prepared in accordance with standard techniques known to one of skill in the
17 art. For example, Chapter 85 entitled "Intravenous Admixtures" by Salvatore J.
18 Turco in the Eighteenth Edition of Remington's Pharmaceutical Sciences, Mac
19 Publishing Co. (1990), incorporated herein by reference, provides standard
20 techniques for preparing a pharmaceutically acceptable IV composition useful in
21 accordance with this invention.

22 Additionally, a patient having a medical condition which is found to
23 respond advantageously to CM101 may be treated with a pharmaceutical
24 composition of the present invention. For example, a patient having a tumor may
25 be advantageously treated by intravenously administering the pharmaceutical
26 composition taught herein. U.S. Patent No. 5,010,062 discusses the treatment of
27 certain tumors in humans and is incorporated herein by reference.

28 Quantitative and Qualitative Analysis

29 HPLC Analysis

30 The purity and amount of CM101 obtained from a sample after HIC
31 chromatography is established by high pressure liquid chromatograph (HPLC) gel
32

1 filtration analysis. The gel filtration column is typically equilibrated with 10%
2 acetonitrile in water and the biologically active CM101 is eluted as an included
3 homogeneous narrow peak. Alternatively, the column may be developed in 10mM
4 phosphate buffer, pH 8.4, which yields a more included peak. An ammonium
5 acetate (NH₄OAc) buffer, pH 8.4, may be used as a further alternative to the
6 10mM phosphate buffer.

7 A typical detector response (UV 203 absorption) using 30, 50, and 100µg
8 pure CM101 standards injected in 100µl of developing buffer on a Hydragel 1000
9 column (Waters, Millford, MA) is 26×10^6 , 48×10^6 , and 97×10^6 area units,
10 respectively which yields a dose response curve for quantitation of unknown
11 samples.

12 The molecular weight of CM101 may also be measured by gel filtration
13 chromatography. A non-denaturing buffer such as the acetonitrile, phosphate, or
14 ammonium acetate buffers described above are used to run the column. The
15 CM101 elution is compared to that of standard dextran polysaccharide markers of
16 different molecular weight. The CM101 has a molecular weight of approximately
17 300,000 Daltons under these conditions.

18
19

20 Amino Acid Analysis

21 Quantitative and qualitative automated amino acid analysis may be
22 performed with standard commercially available equipment. e.g., PicoTag,
23 available from Waters, Millford, MA.

24

25 ANA-1 Assay

26 To monitor the biological activity of the different fermentation and
27 purification steps, an *in vitro* assay employing a transformed mouse macrophage
28 cell line may be used. The assay measures IL-6 production of the mouse
29 macrophage ANA-1 in response to CM101 exposure.

30 Particularly, CM101 induces raf/myc transformed murine bone marrow
31 macrophage cell line ANA-1 to respond *in vitro* by IL-6 production. Other
32 macrophage-like cell lines and fresh peripheral blood leukocytes can also be used.

To perform the ANA-1 assay, samples are first diluted to the appropriate range (depending on the expected level of CM101 activity) and four to eight concentrations are tested at 1:4 dilutions. A CM101 standard curve using clinical grade CM101 reconstituted in PBS is generated. A 4000 ng/ml solution, which gave a 2000 ng/ml final concentration after the cells were added, was made in PBS, along with six serial 1:2 dilutions. Cells at a concentration of 2×10^6 /ml may be used, for example. Sensitivity of the assay was increased by adding 200 U/ml murine IFN- γ to the ANA-1 cells. Final cultures were 100 U/ml IFN- γ .

The microliter plate with cultures should be placed in a 37°, 5% CO₂-in-air, humidified incubator overnight (16 - 18 hours), and then be followed by an ELISA IL-6 Assay (R.D. Systems, Minneapolis, MN). Specifically, culture supernatants are transferred to the IL-6 assay plate and the plate is held at 4°C until the IL-6 assay is complete.

Dot Blot Assay

An alternative rapid procedure to quantitatively detect CM101 in solutions or biological fluids is to blot samples on polyvinylidene difluoride (PVDF) membranes in serial dilutions. The amount of CM101 is quantitated using either a fluorescently-tagged mouse monoclonal antibody to CM101 or a mouse monoclonal antibody to CM101 followed by a fluorescently-tagged anti-mouse IgG. Antibody 7A3 directed against CM101 antigen is useful for this purpose. Quantities of CM101 in the different fractions are established by comparison to a standard curve of serial diluted CM101 standard.

Sheep Pulmonary Arterial Pressure Assay

The toxin affects sheep lungs by increasing pulmonary hypertension, manifested by increased pulmonary arterial pressure and by increased lung vascular permeability.

CM101 samples in phosphate buffered saline (PBS) may be administered to lambs by infusion and changes in pulmonary arterial pressure recorded at 15 minute intervals. These changes in pressure are correlated to CM101 activity. (Hellerqvist, C.G. et al., *Studies on group B β -hemolytic streptococcus I. Isolation*

1 *and partial characterization of an extra-cellular toxin., Pediatr. Res., 15:892-898*
2 (1981)).

4 Sugar Analysis

5 A 100 μ g quantity of a sample is hydrolyzed for two hours at 100°C in a
6 mixture of trifluoroacetic acid (TFA), acetic acid (HOAc) and water in a ratio of
7 5:70:25. The solution is evaporated and the sample is further hydrolyzed for two
8 hours at 100°C in a mixture of TFA and water in a ratio of 2:8. This process
9 completely hydrolyses all glycosidic linkages in the sample. The N-acetyl groups
10 originally present on the amino sugars are also removed.

11 The samples are then analyzed on the Dionex sugar analysis system using a
12 PAD (Pulsed Amperometric Detection) detector. The resolution is illustrated in
13 Fig. 4.

14 The purity of the sample is established by quantitative and qualitative sugar
15 analysis. The principle is illustrated in Figs. 3 and 4. A sample of
16 polysaccharide quantitated by HPLC is supplemented with an internal standard 6-
17 deoxy-D-glucose hydrolyzed and analyzed. The method described in this section
18 gives a linear dose response in the range tested and qualitative analysis is
19 accomplished by comparing retention times of unknowns with the standards.

22 EXAMPLES

24 **Example 1: A scaled-up purification scheme for CM101**

25 A Group B Streptococcus Serotype III isolate working stock was used in
26 conjunction with a 3,000 gallon fermentor. A 25 ml seed of the bacterial culture
27 is used for an 80 liter vessel with a 65 liter working volume (lwv) which is then
28 used to inoculate a 750 lwv vessel, and which, in turn, goes into the final 7500
29 lwv (3,000 gallon) fermentor. Alternatively, the 65 lwv may be used to inoculate
30 the 7500 lwv fermentor directly.

1 The cultures are terminated at late log phase by autoclaving. The bacteria
2 are then removed by continuous centrifugation at 10,000 x g, followed by 0.45
3 micron cassette filtration (Millipore Corporation, Bedford, MA).

4 The resulting culture supernatant is then concentrated 15-fold through
5 cassette filtration using 10kD to 50kD cut off cassettes (Millipore) to 500 liters.
6 The concentrated material is then made 2M in salt, preferably sodium phosphate,
7 pH 7.4 (loading buffer) by dialysis.

8 The concentrated supernatant is then subjected to hydrophobic interaction
9 chromatography, through the use of a 60 liter n-butyl Sepharose column
10 (Pharmacia, Uppsala, Sweden) using a BioPilot system (Pharmacia). The capacity
11 of the n-butyl Sepharose resin for the biologic CM101 activity in the media
12 concentrate with no flow-through of activity is approximately 80 liter of media to
13 one liter of resin. After the concentrated supernatant is loaded onto the column,
14 the column is washed with the loading buffer followed by 0.5-1M and then 0.25M
15 phosphate buffer, pH 7.4. The CM101-containing fraction is eluted with water in
16 approximately 120 liters or two column volumes and concentrated to 2 liters in a
17 cut-off cassette in the range of 10kD to 50kD. The column elution is controlled
18 by a preestablished program in the BioPilot and the eluate is monitored by UV
19 absorption at 206 and 280 nm, conductivity, and pH.

20 The CM101-containing 2 liter fraction is dialyzed against 0.05M saline, pH
21 7.0 and then heated to the range of 75 - 80°C and 0.2-2 liters of phenol are
22 added. The mixture is then heated to 80°C and maintained at that temperature for
23 5 minutes. Following this, the mixture is chilled to 4°C. The water phase
24 resulting from this step is preferably extracted twice with 0.2 volumes chloroform
25 before application to a DEAE Sephacel FF column (Pharmacia, Uppsala, Sweden)
26 equilibrated in water. The column is washed with 100mM saline, 0.05M NaOAc,
27 pH 7.4, and the biologically active material, CM101, is then eluted from the
28 DEAE column with a NaCl gradient. The biological activity is detected by Il-6
29 assay and HPLC analysis. The quality of the CM101 purified through this
30 procedure is established by HPLC and sugar analysis as well as biological activity
31 assays by Il-6 and sheep tests.

This scaled up purification scheme provides the advantage of avoiding the large volume, early phenol-water extraction procedure of the alcohol precipitate used in the previous procedure.

Results

Figs. 5a-b show elution profiles of a media concentrate on a butyl-Sepharose HIC column in 2M K_2HPO_4 , pH 7.2. The various peaks are the results of timed step-wise changes in the elution gradient. Fig. 5a represents the profile measured at UV 206 absorbance, which quantitates the peak fractions for total organic material, and shows the CM101 in the last narrow peak (approximately 383 minutes). Fig. 5b represents the profile measured at UV 280 absorbance, which quantitates the amount of protein in the different fractions.

By performing the HIC column step, CM101 is caused to bind to the column whereas up to 99.7% of the protein and up to 98.5% of neutral and charged polysaccharides pass through the column, as indicated in Table 1.

Table 1. Purification of CM101 Activity by HIC Chromatography
Quantitation by Integration of UV 280 and 206 Profiles

<u>Final Elution Possible Protein UV280</u>			<u>Total Organic</u>
<u>UV206</u>			
		<u>Recovered %</u>	<u>Recovered %</u>
AP 6P6	Water	0.85	2.67
AP 2P9	Water	1.08	0.19
10K5P6	Water	0.82	1.05
10K5P6	Water	0.46	2.43
AP 1 P9	Buffer A	0.39	1.90
10K5P6	Buffer A	0.50	1.51
AP 6P6	Buffer A	0.19	1.35

In Table 1, different fermentation lots as alcohol precipitates (AP), AP1, AP2, and AP6, and 10k concentrates were subjected to HIC chromatography and eluted with either water or Buffer A. Both processes yield approximately the same

1 efficacious removal of exogenous and endogenous protein (UV 280) and
2 polysaccharides and general organics (UV 206).

3 Figs. 6a-b present an HPLC profile, and a Diodo-Ray spectrum, of an HIC-
4 purified water-eluted fraction containing CM101 and monitored at UV 203
5 absorbance. These figures illustrate the minimal presence of 260 absorption
6 (RNA and DNA) and 280 absorption (protein) for the CM101 containing peak.

7 After the HIC fraction is further subjected to the phenol/saline extraction and
8 ion exchange steps, the purity of the HIC water-eluted peak is further improved,
9 as seen in Figs. 7a-b. Note the narrow symmetric peak at approximately 16
10 minutes from time zero and the lack of absorption at 260 (RNA/DNA) and 280
11 (protein). For the elution profiles shown in Figs. 6a-b and 7a-b, the HPLC was
12 performed with 10% acetonitrile in water and the flow rate was approximately 0.3
13 ml/min.

14 These elution profiles as well as the biological activity are similar to those
15 obtained when the alcohol precipitate is used as the starting material for the HIC
16 column.

17 The ability of the HIC fractions from the 10k starting material to induce IL-6
18 synthesis in ANA-1 cells is illustrated in Fig. 8. HIC chromatography yielded an
19 approximate recovery of 50% of the total biologic activity in the media
20 supernatant as measured by an ANA-1 Assay. Dot blot assays of the same
21 material which show immunoreactivity in the presence of CM101 antigen were
22 used to confirm ANA-1 assay results.

23 The different fractions obtained from the 10k concentrate after HIC
24 chromatography were also tested in the sheep model for biologic activity. The
25 amount of CM101 activity is determined based on a dose response curve using
26 current clinical CM101 (1 Unit of activity corresponds to 7.5µg/kg). The results
27 are shown in Table 2 wherein HIC fractionations of alcohol precipitate (AP) and
28 media concentrate (10k) are compared.

Table 2: Amount of CM101 Obtained from HIC Chromatography of AP and 10K Material Based on Quantitation of Biological Activity in Sheep Model

<u>Fraction</u>	Alcohol Precipitate (AP)	Media Concentrate
	<u>CM 101 Activity $\mu\text{g/l}$</u>	<u>CM 101 Activity $\mu\text{g/l}$</u>
Pre-Load	466	Not Available
1M Phosphate	118	209
0.25 Phosphate	28	2970
Water	225	7520

The biological activity of CM101 as purified by the method of the present invention was also measured with the pulmonary arterial pressure assay in sheep, and then compared with the activity of CM101 purified by the old process, for example as taught in U.S. Patent No. 5,010,062. The material purified according to the invention exhibited a specific activity of two to three times greater than material which was purified by the old process, that is, which had not been contacted with an HIC resin.

The product yield of the method of the present invention is also evidenced above, as the known methods provide about 300 μg of CM101 per liter of fermentation volume, as compared with the 7520 $\mu\text{g/l}$ value shown above.

The purified CM101 illustrated in Figs. 7a-b obtained by the process of the present invention was also subjected to sugar analysis. The sugar yields are shown in Fig. 9.

Quantitatively, the CM101 obtained by the method of the present invention is greater than 95% pure carbohydrate and contains less than 5% of protein established by quantitative and qualitative as presented above and by automated amino acid analysis (PicoTag, Waters, Millford, MA).

Example 2: Comparison of Current Clinical Grade and New Composition

The CM101 obtained by the method of the present invention is improved over the current clinical grade CM101. Particularly, the HPLC elution profile of Fig.

10 as compared with Fig. 7a illustrates higher purity in the sample produced according to the present invention. Fig. 7a shows one narrow and symmetric main peak, instead of several peaks.

To further demonstrate the advantageous use of the HIC column and to provide further evidence of purification of the toxin known as CM101, current clinical grade CM101 was subjected to an HIC column and HPLC purification and a sugar analysis was performed. The results, in Fig. 11, may be compared to Fig. 9. The sugar analysis shows quantitatively and qualitatively similar end products, and demonstrates that the HIC chromatography process removes sugars not related to biologically active CM101. This result is borne out in Table 3, as well.

Table 3: Carbohydrate Composition of CM101 (Presented as Integral Carbohydrate Ratios)

	CM101 Purified With Present Method	Current Clinical Grade CM101	Current Clinical Grade CM101 Further Purified
Sugar			
Rhamnose	0	3	0
Mannose	1	3	1
Galactose	3	24	3
Glucose	1	7	1
Glucosamine*	1	13	1
Galactosamine*	1	5	1

*Present as "N-acetyl-glucosamine and N-acetyl-galactosamine", respectively, in the native polysaccharide.

The table of sugar residues presented above gives approximate molar ratios. The actual residues of the CM101 purified according to the present method (the

1 first column) are in a range of (0.2-1 mannose):(2.5-3.5 galactose):(0.5-1
2 glucose):(1 N-acetyl glucosamine):(0.5-1 N-acetyl galactosamine). The numbers
3 are normalized to N-acetyl glucosamine; thus N-acetyl glucosamine is set at 1.

4 For further comparison, Figs. 12a-b show HPLC profiles of CM101
5 manufactured by the old process (Fig. 12a) and according to the method of the
6 present invention (Fig. 12b). The gel filtration column (Ultrigel 100, Waters,
7 Milford, MA) was developed in 10mM phosphate buffer, pH 8.4. As seen in Fig.
8 12b, the CM101 purified according to the method of the invention elutes in a
9 relatively narrow peak over an approximately 5 minute range. The elution time is
10 approximately 24 minutes from time zero with a 0.3 ml/min flow rate. The
11 material purified by the old process, by contrast, elutes in a broad peak over
12 approximately 12 minutes.

13 14 **Example 3: Analysis of CM101 by SDS-PAGE/Western Blot**

15
16 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed
17 using a 4-20% gradient gel. CM101 samples in a buffer of 2% SDS, 0.5 M Tris-
18 HCl, 5% glycerol, 0.05% bromophenyl blue, and 5% β -mercaptoethanol pH 0.8
19 were incubated at 55° for 10 minutes and applied in a 4% stacking gel to the 4 to
20 20% SDS-PAGE running gel. The gel was run at 200 Volts for 90 minutes.

21 The gel was developed in a western blotting buffer (25 mM Tris, 192 mM
22 glycine and 20% Methanol) and blotted at 100 Volts for 2 hours. The gel was
23 blocked with 5% fat-free milk in PBS at room temperature for 1 hour and washed
24 twice with a binding buffer (phosphate buffered saline, 2% fetal bovine serum,
25 and 0.5% TWIN-20 detergent (BBT)), then incubated 1 hour with 10 μ g/ml of
26 7A3 (monoclonal antibody to CM101).

27 The gel was incubated 4 times for 10 minutes with BBT and incubated with
28 the alkaline phosphatase conjugated anti-mouse antibody for 45 minutes, washed 4
29 times with BBT and developed with Pierce Single-Step AP developer for 50
30 minutes.

1 The SDS-PAGE/Western Blot analysis suggested that CM101 has a
2 component of 26,000 Daltons or a multiple thereof when analyzed under these
3 conditions.

4 Thus, the method of the present invention provides an improved method of
5 purification which minimizes the difficulty of hazardous steps and provides
6 excellent purity. Additionally, the product produced by the method taught herein
7 is improved over the currently available CM101.

8 All publications and patent applications mentioned in this specification are
9 herein incorporated by reference to the same extent as if each individual
10 publication or patent application was specifically and individually indicated to be
11 incorporated by reference.

12 The invention now being fully described, it will be apparent to one of
13 ordinary skill in the art that many changes and modifications can be made thereto
14 without departing from the spirit or scope of the appended claims.

1 WHAT IS CLAIMED IS:

2

3 1. A method of purifying a polysaccharide toxin from GBS bacteria, which
4 method comprises contacting an aqueous mixture containing the toxin with an HIC
5 resin.

6

7 2. The method of claim 1 wherein the HIC resin contacting step further
8 comprises separating the toxin from the HIC resin.

9

10 3. The method of claim 1 wherein the toxin is CM101.

11

12 4. The method of claim 1 wherein the purity of the toxin resulting from the HIC
13 resin contacting step is increased approximately 100-500 fold.

14

15 5. The method of claim 1 wherein the toxin resulting from the HIC resin
16 contacting step is greater than 40% pure.

17

18 6. The method of claim 5 wherein the toxin resulting from the HIC resin
19 contacting step is at least approximately 60% pure.

20

21 7. The method of claim 6 wherein the toxin resulting from the HIC resin
22 contacting step is at least approximately 90% pure.

23

24 8. The method of claim 1 wherein the toxin resulting from the HIC resin
25 contacting step is at least approximately 95% pure.

26

27 9. The method of claim 1 wherein the HIC resin further comprises a resin
28 having interactive groups selected from the group consisting of alkyl, alkoxy, and
29 aryl groups.

30

31 10. The method of claim 9 wherein the interactive group are alkyl groups having
32 in the range of 2 to 12 carbons.

- 1 11. The method of claim 10 wherein the interactive groups are butyl groups.
- 2
- 3 12. The method of claim 11 wherein the interactive groups are normal butyl
- 4 groups.
- 5
- 6 13. The method of claim 9 wherein the interactive group is a phenyl group.
- 7
- 8 14. The method of claim 1 further comprising extracting the toxin with an
- 9 aqueous phenol mixture.
- 10
- 11 15. The method of claim 1 further comprising contacting the toxin with an ion-
- 12 exchange resin.
- 13
- 14 16. The method of claim 1 further comprising after the HIC resin contacting step,
- 15 extracting the toxin with an aqueous phenol mixture, and
- 16 contacting the toxin with an ion-exchange resin.
- 17
- 18 17. A toxin from GBS bacteria purified by the method of claim 1.
- 19
- 20 18. The toxin of claim 17 wherein the toxin is CM101.
- 21
- 22 19. The toxin of claim 18 wherein the toxin is at least approximately 60% pure.
- 23
- 24 20. The toxin of claim 19 wherein the toxin is at least approximately 90% pure.
- 25
- 26 21. The toxin of claim 20 wherein the toxin is at least approximately 95% pure.
- 27
- 28 22. The toxin of claim 18 further comprising sugar residues of mannose,
- 29 galactose, glucose, N-acetyl glucosamine, and N-acetyl galactosamine in a molar
- 30 ratio of (0.2-1 mannose):(2.5-3.5 galactose):(0.5-1 glucose):(1 N-acetyl
- 31 glucosamine):(0.5-1 N-acetyl galactosamine).
- 32

- 1 23. The toxin of claim 22 wherein the sugar residues of mannose, galactose,
2 glucose, N-acetyl glucosamine, and N-acetyl galactosamine are present in a molar
3 ratio of (1 mannose):(3 galactose):(1 glucose):(1 N-acetyl glucosamine):(1 N-acetyl
4 galactosamine).
5
- 6 24. The toxin of claim 18 for use in a method of therapy.
7
- 8 25. The toxin of claim 18 having a molecular weight of approximately 300,000
9 Daltons as measured by gel filtration chromatography in non-denaturing
10 conditions.
11
- 12 26. The toxin of claim 18 characterized in that the toxin elutes in a narrow
13 symmetrical peak measured at 203 nm absorbance at approximately 16 minutes
14 from time zero when analyzed by HPLC in a 10% acetonitrile buffer with a flow
15 rate of approximately 0.3 ml/minute.
16
- 17 27. The toxin of claim 18 characterized in that the toxin elutes in a narrow
18 symmetrical peak measured at 203 nm absorbance at approximately 24 minutes
19 from time zero when analyzed by HPLC in a 10 mM phosphate buffer, pH 8.4.
20
- 21 28. The toxin of claim 18 having a specific activity approximately two to three
22 times greater than the specific activity of a GBS toxin that has not had contact with
23 an HIC resin as measured by an assay for increased pulmonary arterial pressure in
24 sheep.
25
- 26 29. A method of purifying a polysaccharide toxin from GBS bacteria comprising:
27 (1) applying a source of GBS bacterial toxin to an HIC column,
28 (2) eluting the HIC column to obtain an HIC eluate including the toxin,
29 (3) extracting the HIC eluate with a combination of phenol and an aqueous
30 solution to form an aqueous phase including the toxin,
31 (4) applying the aqueous phase to an ion exchange column, and
32 (5) eluting the ion exchange column to obtain an ion exchange eluate,

- 1 wherein the ion exchange eluate comprises a substantially pure toxin.
- 2
- 3 30. The method of claim 29 wherein the substantially pure toxin is CM101.
- 4
- 5 31. The method of claim 30 wherein the substantially pure toxin is at least
- 6 approximately 60% pure.
- 7
- 8 32. The method of claim 31 wherein the substantially pure toxin is at least
- 9 approximately 95% pure.
- 10
- 11 33. A composition consisting essentially of substantially pure GBS toxin.
- 12
- 13 34. The composition of claim 33 wherein the substantially pure GBS toxin is
- 14 CM101.
- 15
- 16 35. The composition of claim 34 wherein the toxin is at least approximately 60%
- 17 pure.
- 18
- 19 36. The composition of claim 35 wherein the toxin is at least approximately 90%
- 20 pure.
- 21
- 22 37. The composition of claim 36 wherein the toxin is at least approximately 95%
- 23 pure.
- 24
- 25 38. The composition of claim 34 wherein the toxin further comprises sugar
- 26 residues of mannose, galactose, glucose, N-acetyl glucosamine, and N-acetyl
- 27 galactosamine in a molar ratio of (0.2-1 mannose):(2.5-3.5 galactose):(0.5-1
- 28 glucose):(1 N-acetyl glucosamine):(0.5-1 N-acetyl galactosamine).
- 29
- 30 39. The toxin of claim 38 wherein the sugar residues of mannose, galactose,
- 31 glucose, N-acetyl glucosamine, and N-acetyl galactosamine are present in a molar

- 1 ratio of (1 mannose):(3 galactose):(1 glucose):(1 N-acetyl glucosamine):(1 N-acetyl
2 galactosamine).
3
- 4 40. The composition of claim 34 wherein the toxin has a molecular weight of
5 approximately 300,000 Daltons as measured by gel filtration chromatography in
6 non-denaturing conditions.
7
- 8 41. The composition of claim 34 wherein the toxin elutes in a narrow
9 symmetrical peak measured at 203 nm absorbance at approximately 16 minutes
10 from time zero when analyzed by HPLC in a 10% acetonitrile buffer with a flow
11 rate of approximately 0.3 ml/minute.
12
- 13 42. The composition of claim 34 wherein the toxin elutes in a narrow
14 symmetrical peak measured at 203 nm absorbance at approximately 24 minutes
15 from time zero when analyzed by HPLC in a 10 mM phosphate buffer, pH 8.4.
16
- 17 43. A pharmaceutical composition comprising substantially pure CM101 and
18 a pharmaceutically acceptable carrier.
19
- 20 44. The pharmaceutical composition of claim 43 wherein the composition is at
21 least approximately 60% pure.
22
- 23 45. A method of treating a patient having a medical condition comprising
24 administering the pharmaceutical composition of claim 43 to the patient.
25
- 26 46. A method of treating a patient with a tumor comprising
27 administering the pharmaceutical composition of claim 43 to the patient.
28

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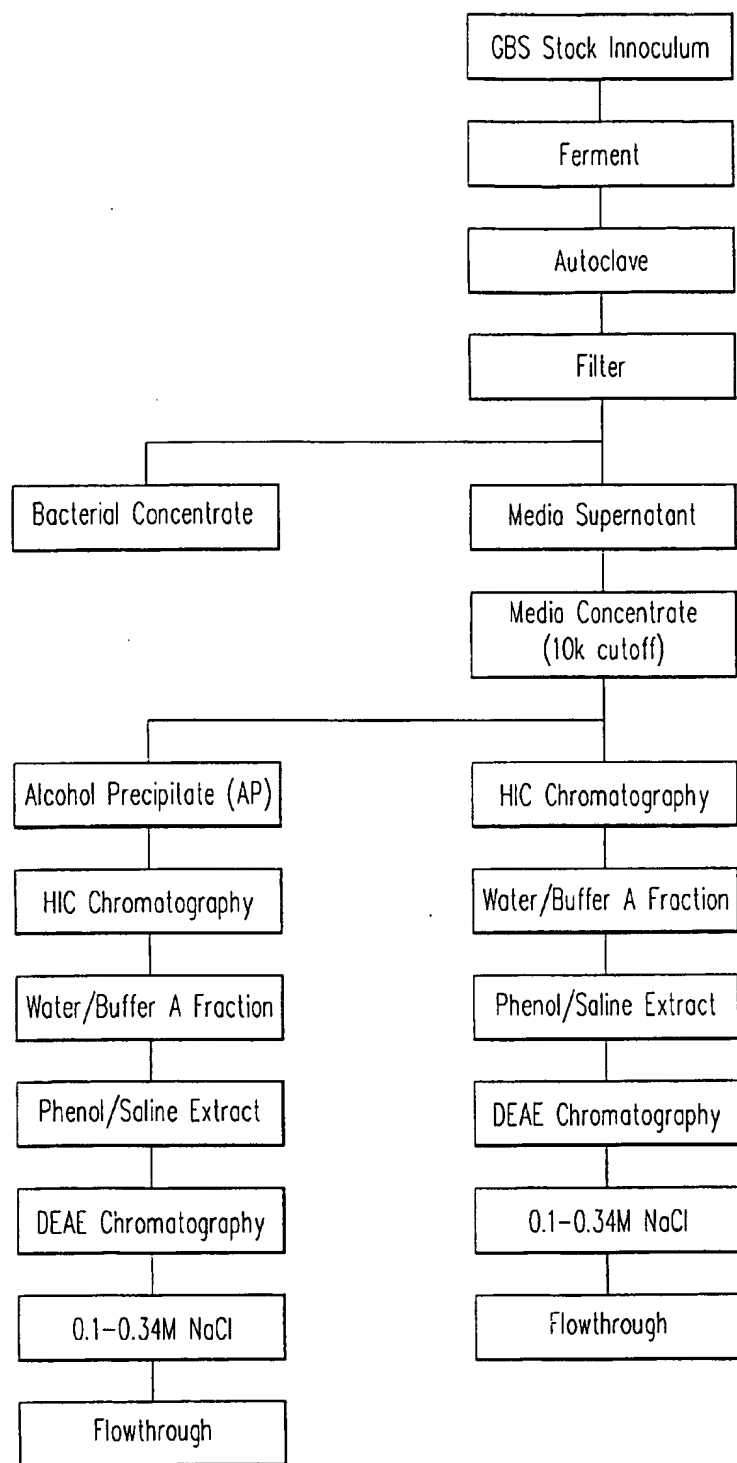


FIG. 1

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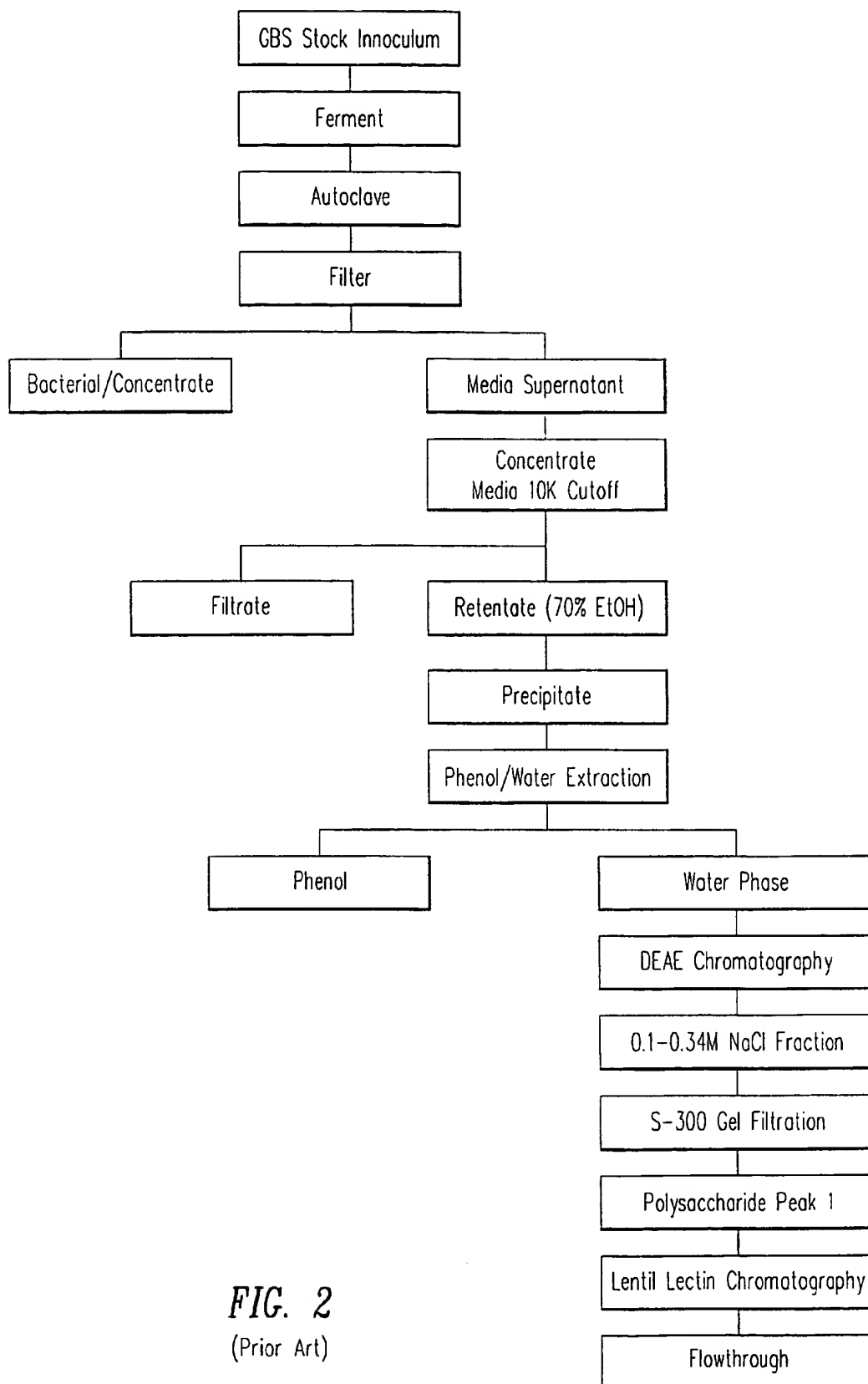
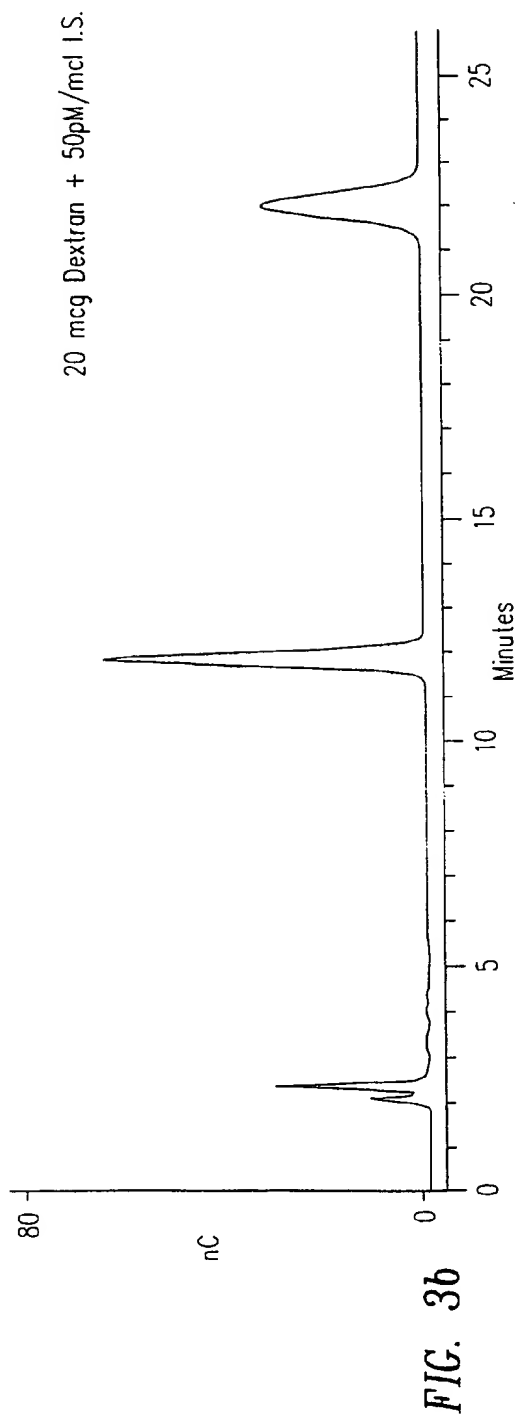
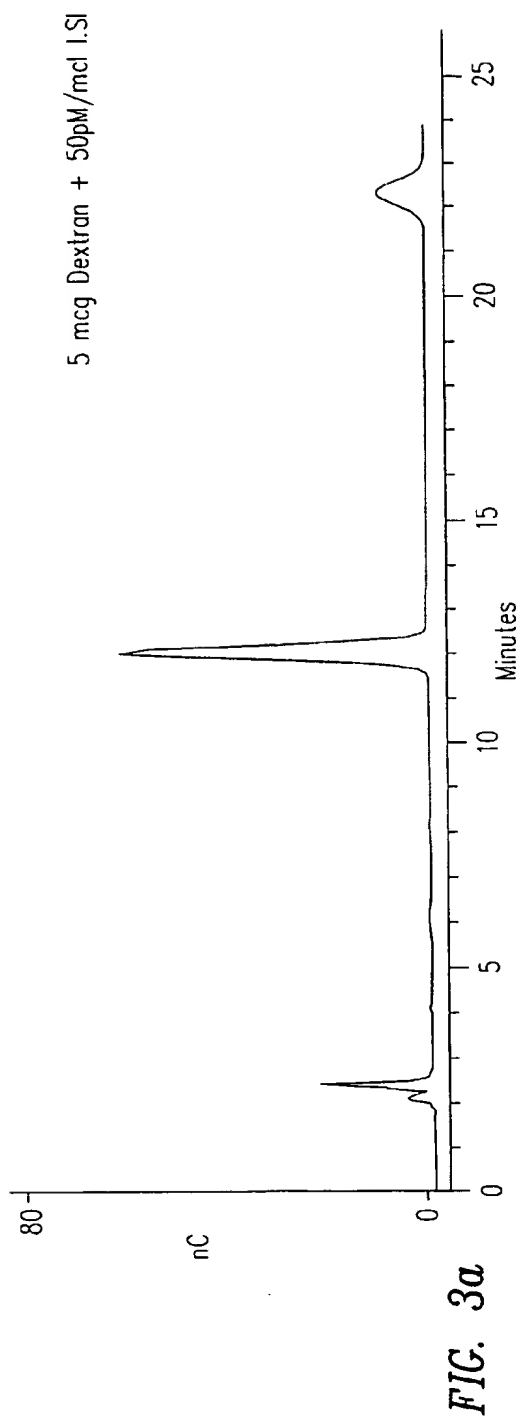


FIG. 2
(Prior Art)

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50 mcg Dextran + 50pM/ml I.S.

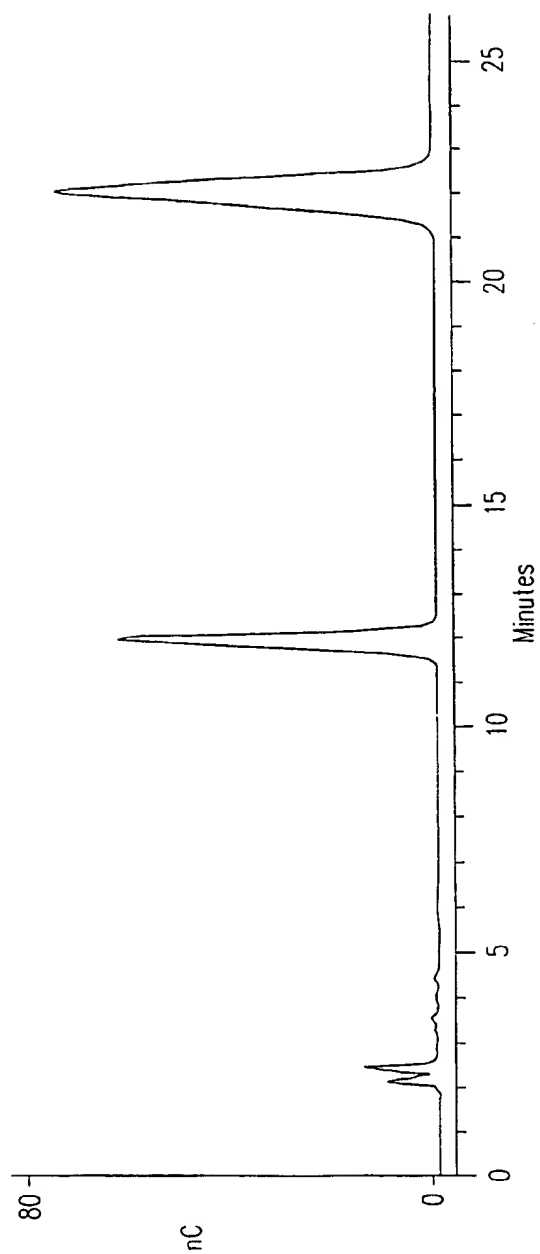


FIG. 3c

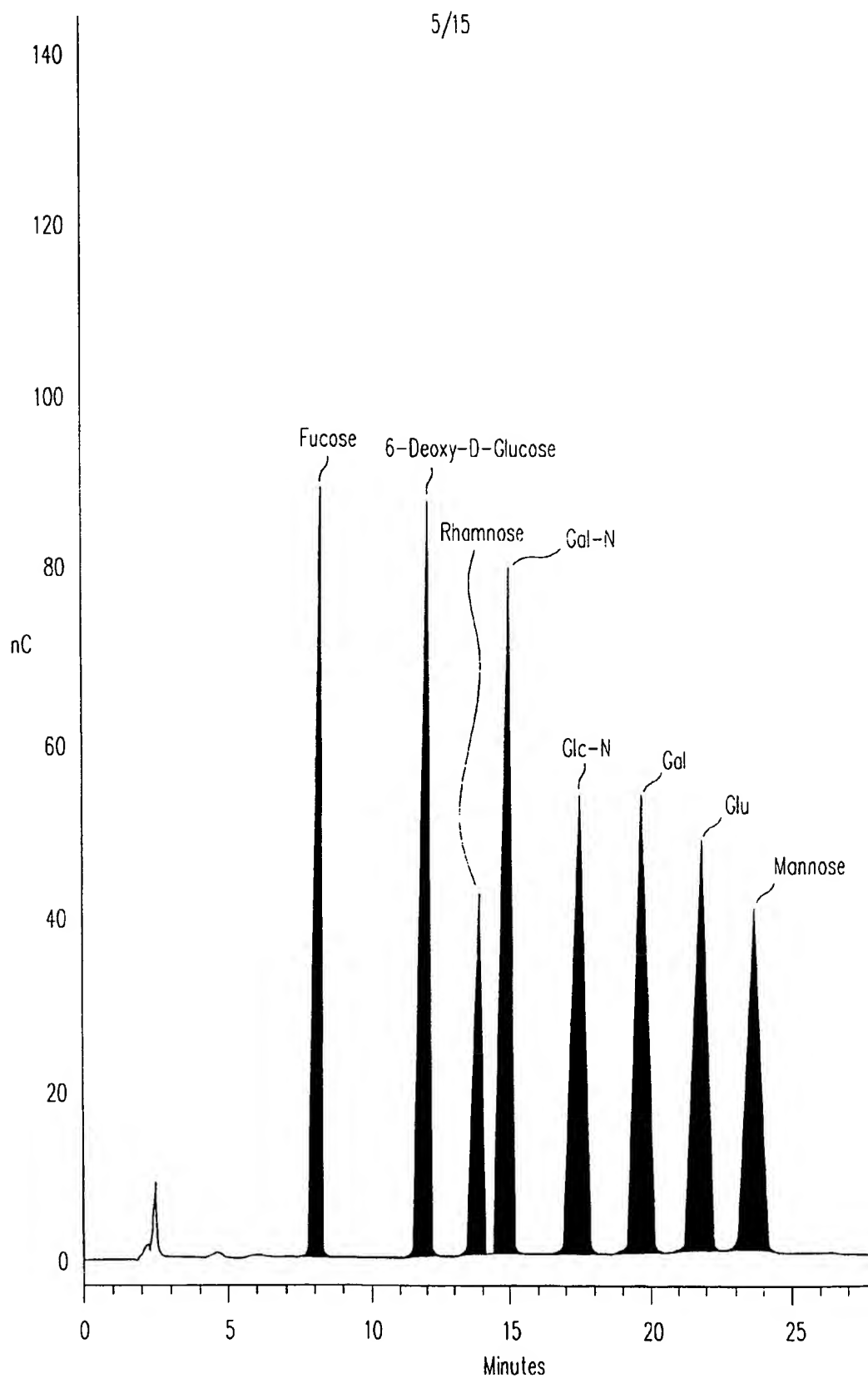


FIG. 4

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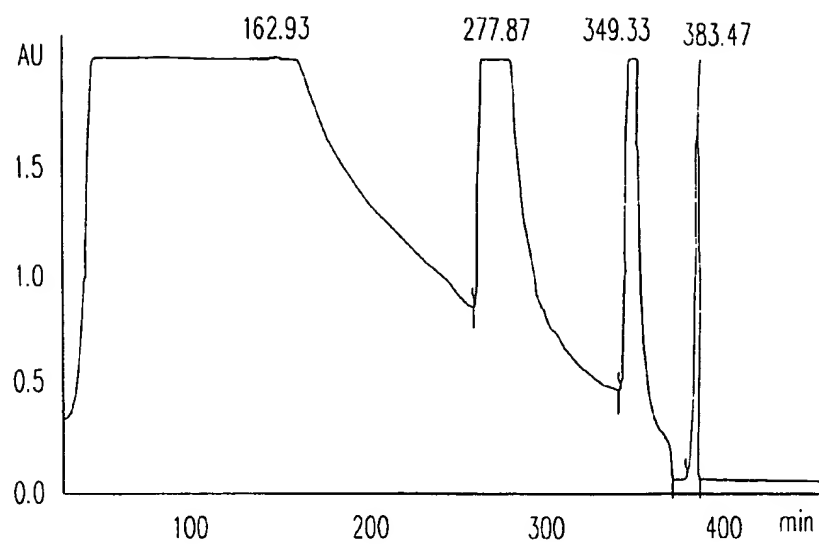


FIG. 5a

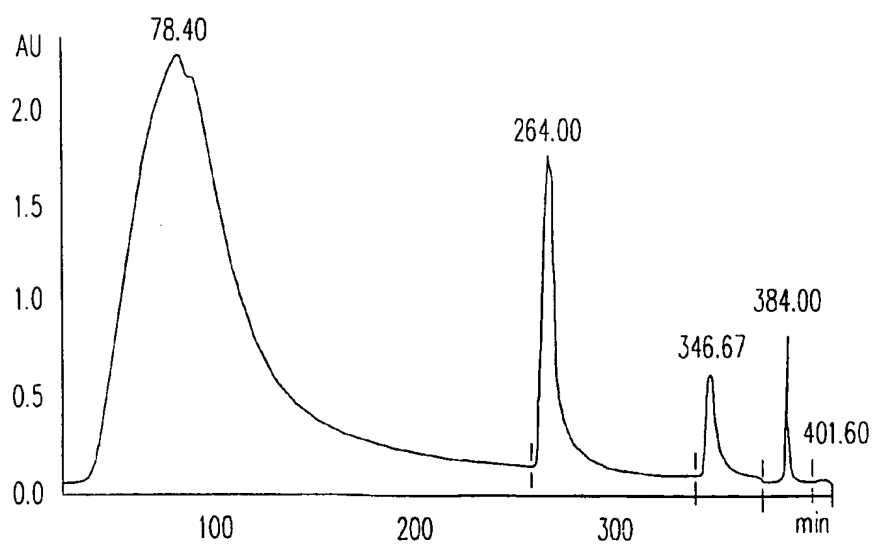


FIG. 5b

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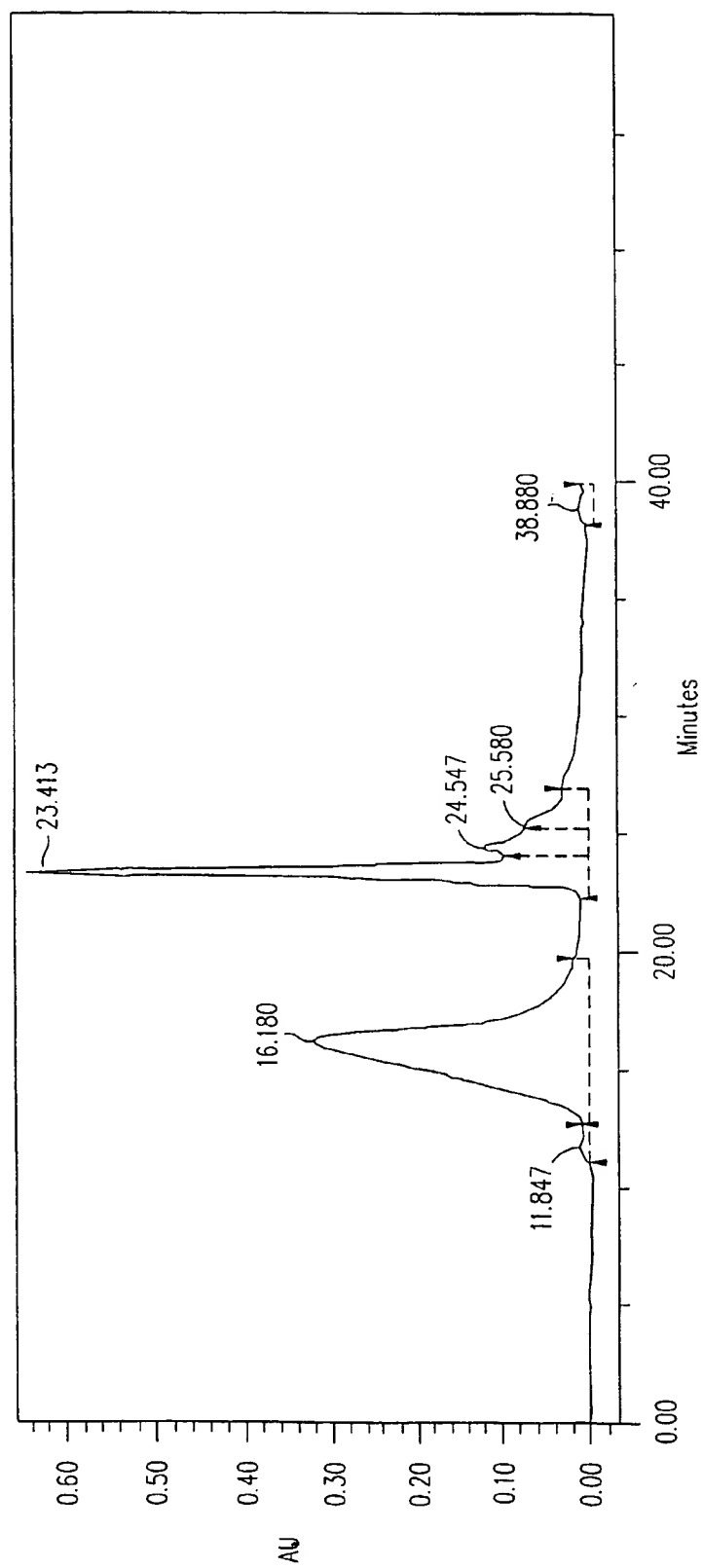
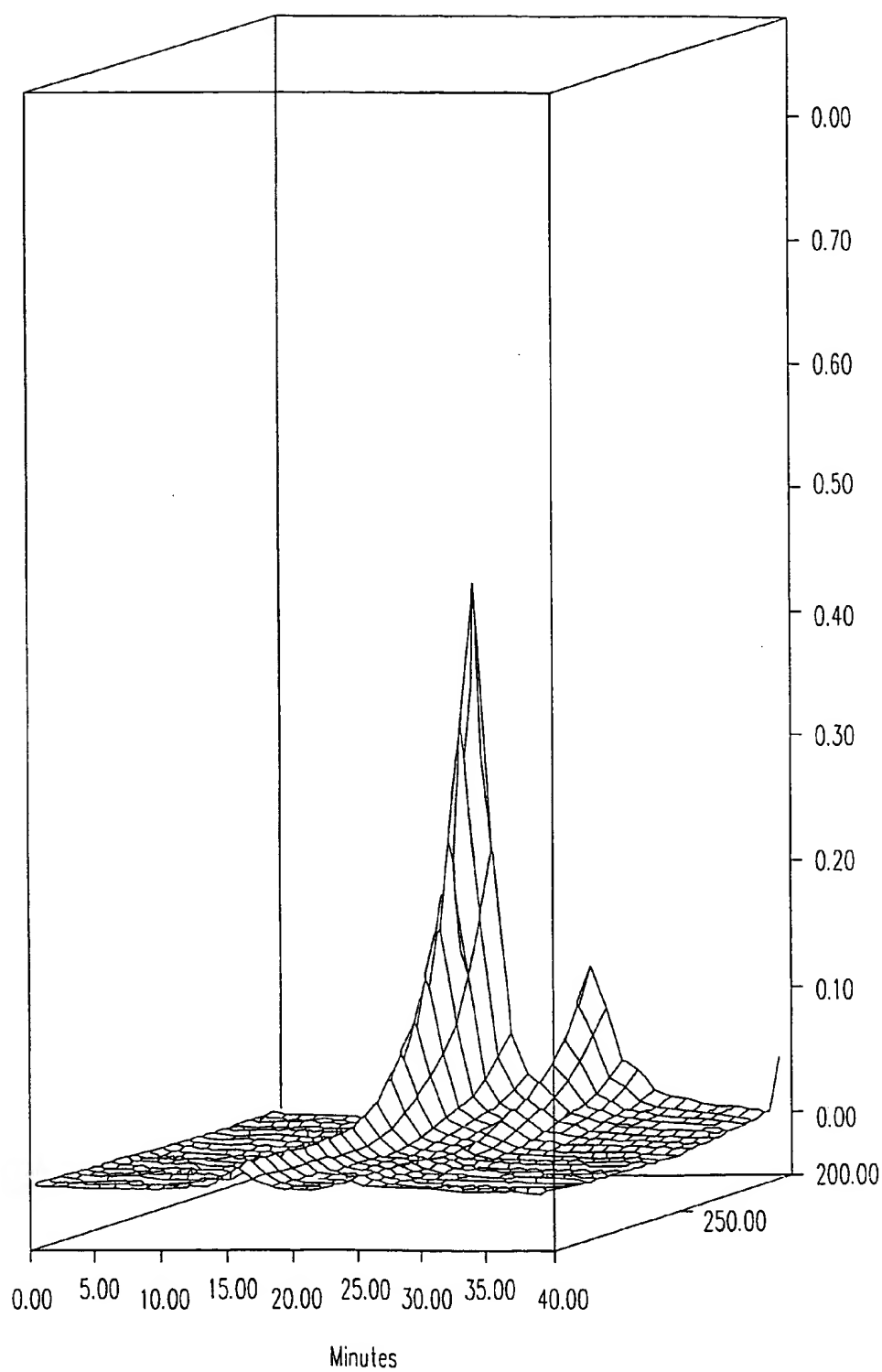


FIG. 6a

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*FIG. 6b*

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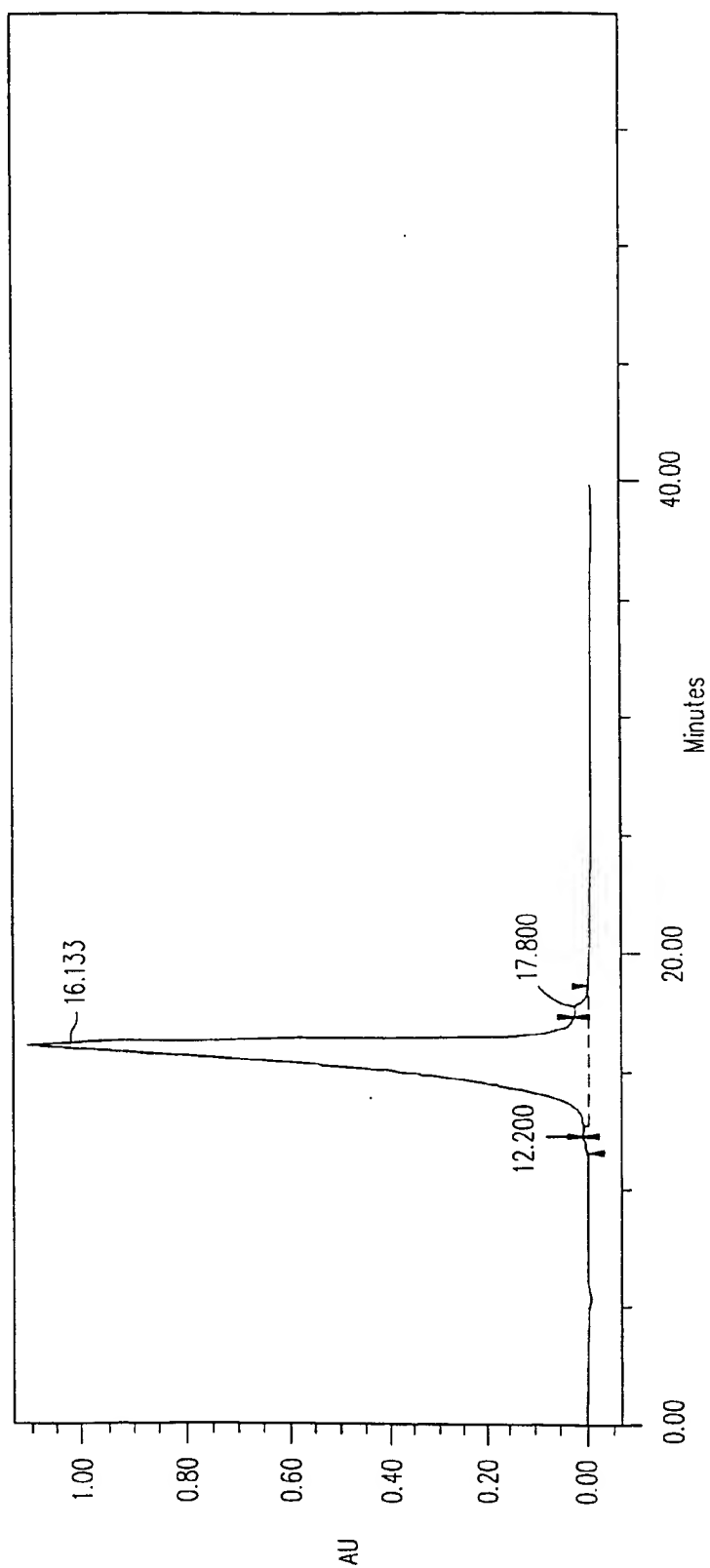


FIG. 7a

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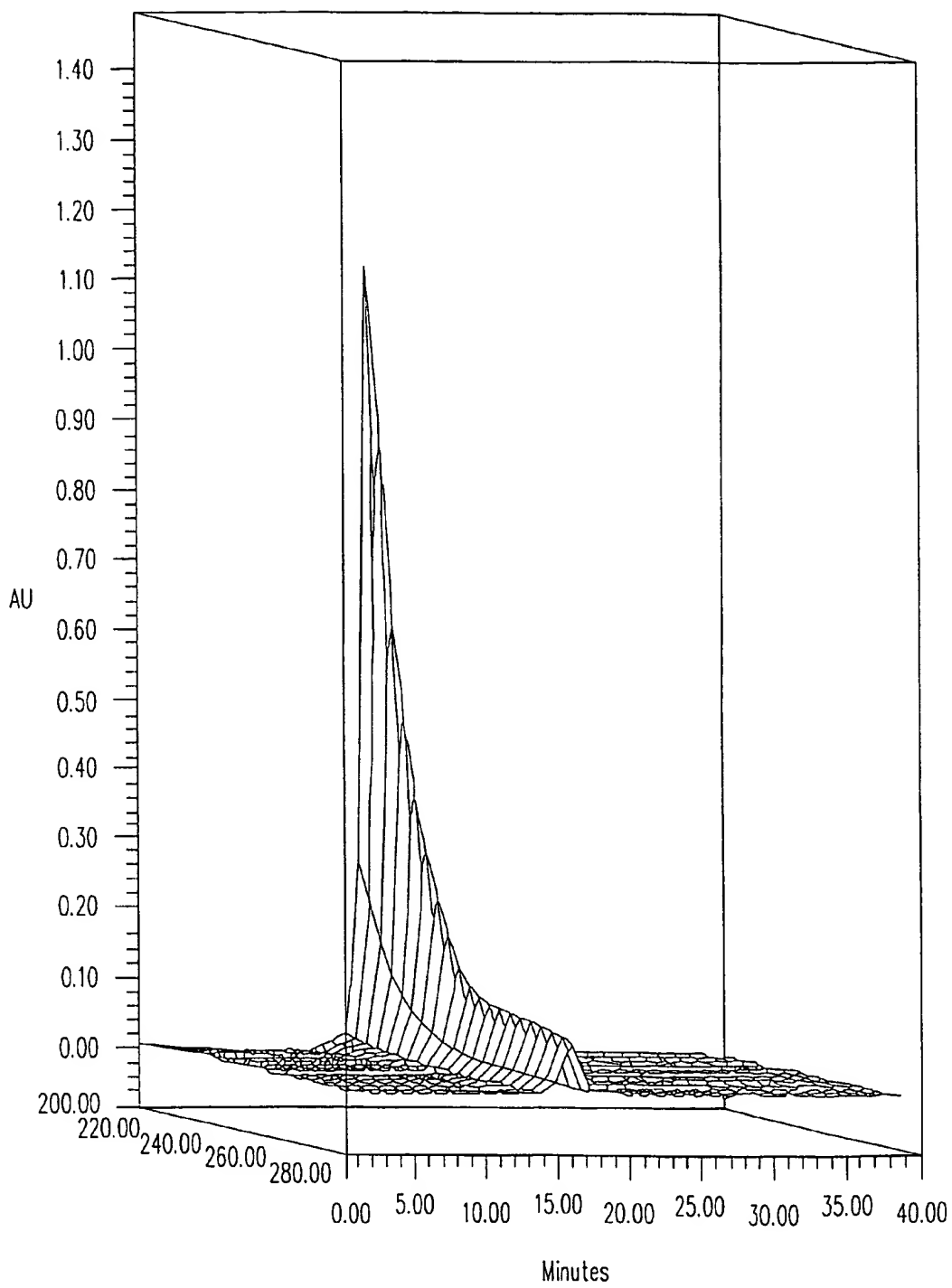
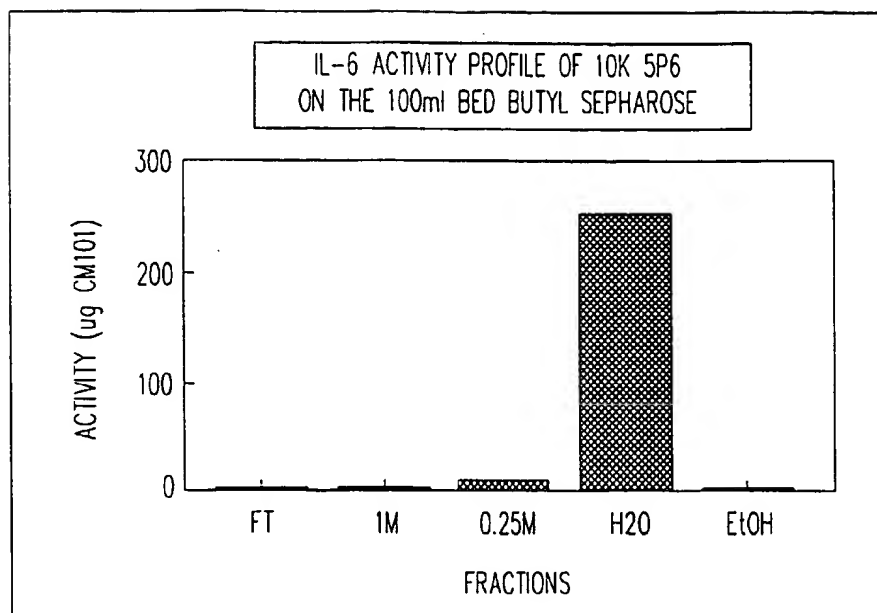


FIG. 7b

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*FIG. 8*

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PK. Num	Ret Time	Component Name	Concentration pmol/ul	Height	Area	Bl. Code	% Delta
1	1.58		0.000	36832	563550	1	
2	3.25		0.000	1869	120205	1	
3	5.42		0.000	43126	510460	1	
4	10.67	Gal-N	2.029	30358	761900	1	-0.03
5	12.58	Glc-N	2.135	25737	718738	2	0.67
6	14.08	Gal	5.363	59697	1781642	2	0.60
7	15.17	Glu	2.417	30036	887398	2	0.57
8	16.17	Man	2.286	15296	581897	2	0.54
9	19.83		0.000	1254	50613	1	
10	24.42		0.000	894	23565	1	
Totals			14.229	245098	5999967		

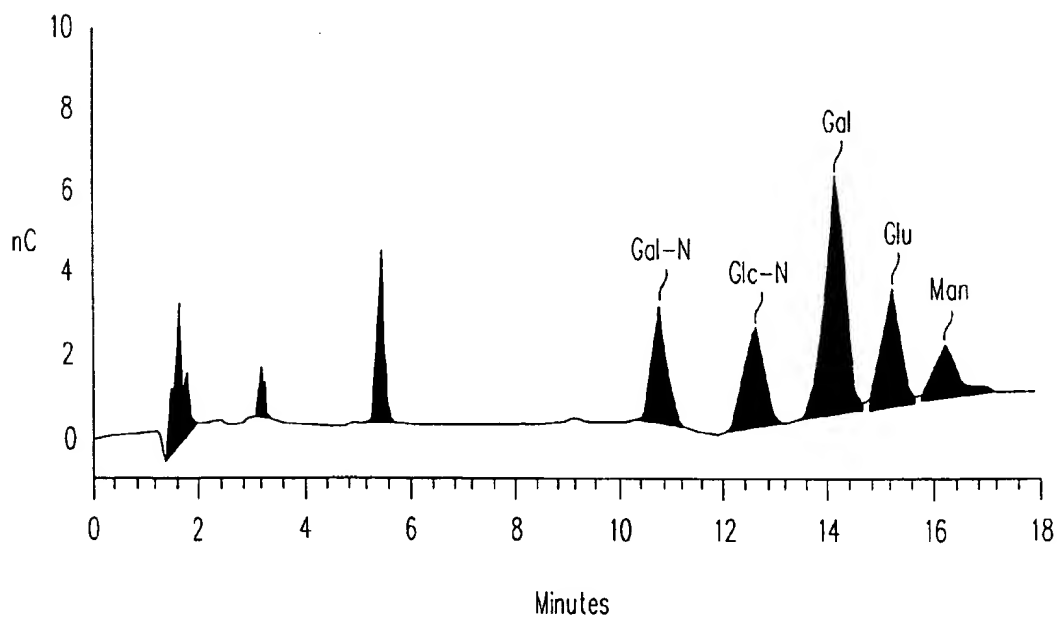


FIG. 9

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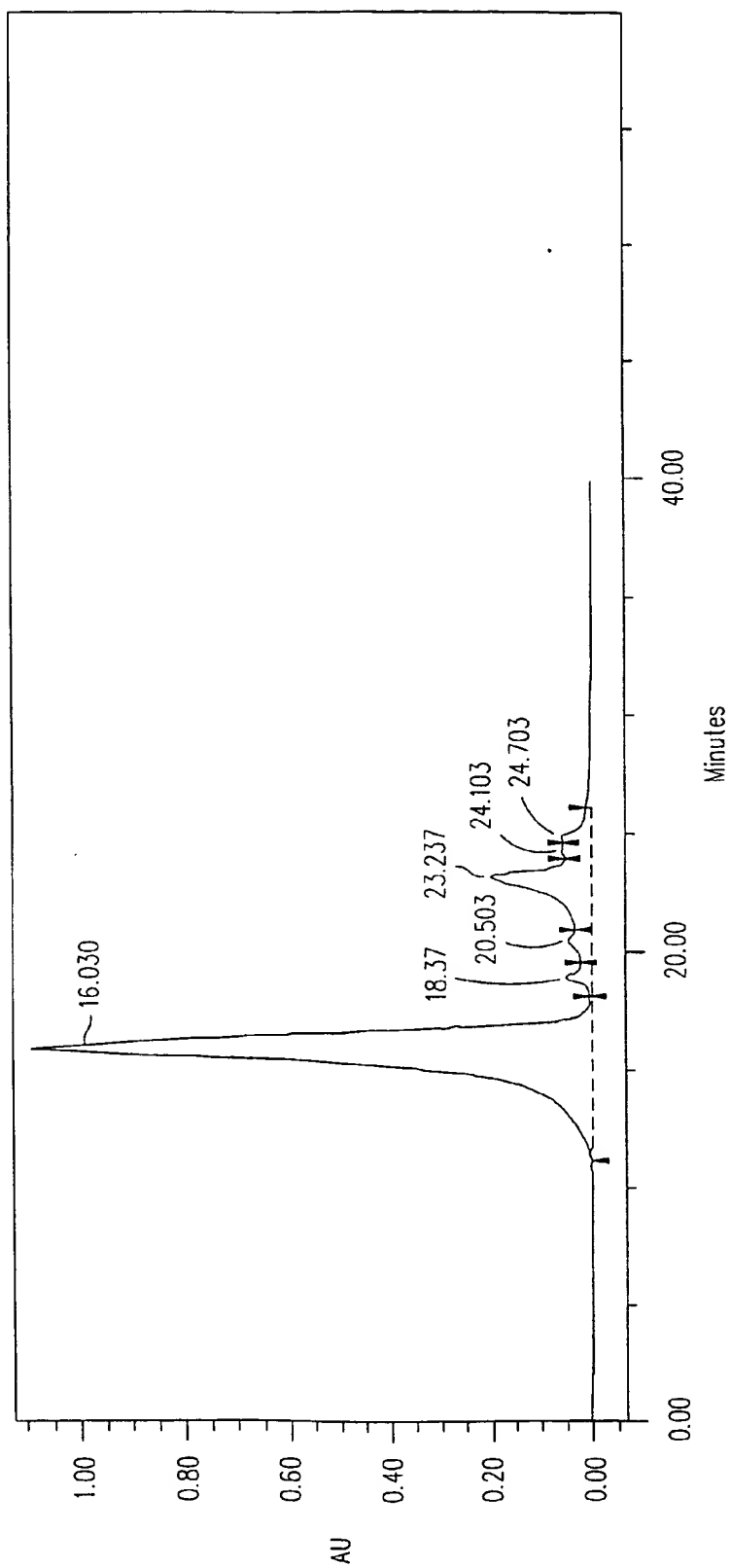


FIG. 10

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PK. Num	Ret Time	Component Name	Concentration pmol/ul	Height	Area	Bl. Code	% Delta
1	1.58		0.000	8497	205165	1	
2	5.33		0.000	28988	380255	1	
3	10.50	Gal-N	0.895	15216	400665	1	0.77
4	12.42	Glc-N	0.783	10964	310639	2	1.36
5	13.67	Gal	2.461	34184	981936	2	-1.18
6	14.83	Glu	1.013	11919	368215	2	-1.11
7	15.92	Mon	0.711	4252	221879	2	3.83
8	23.75		0.000	1781	87055	1	
Totals			5.864	115801	2955809		

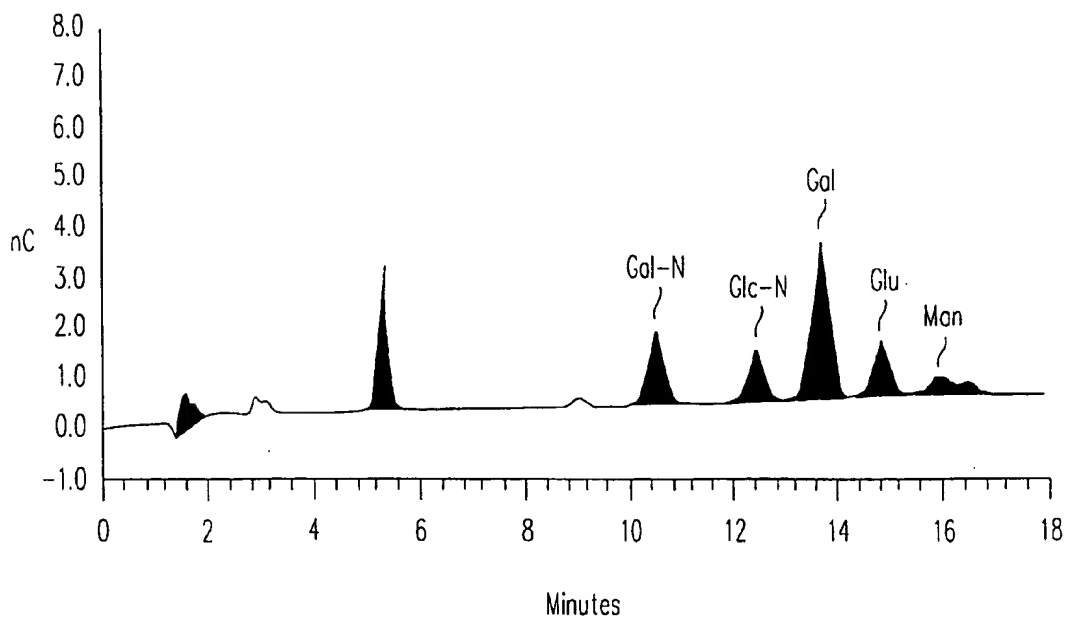


FIG. 11

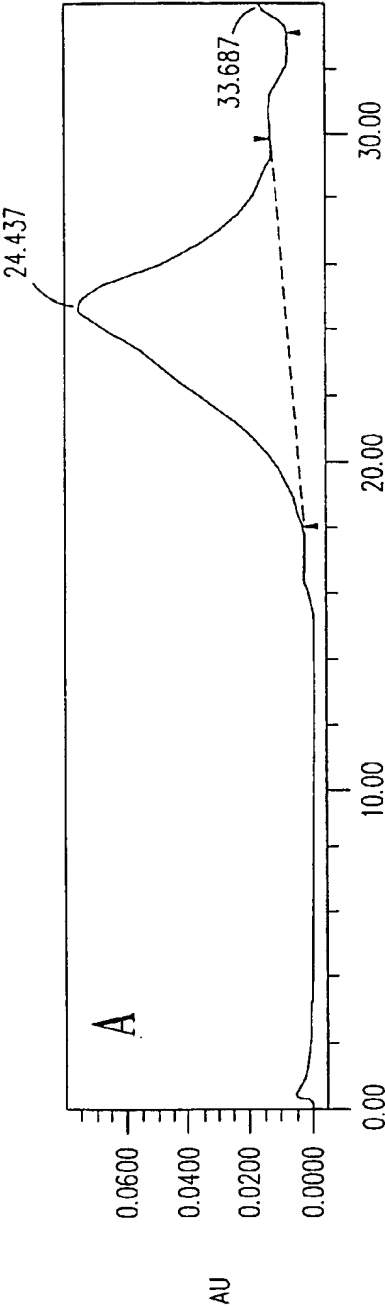


FIG. 12a

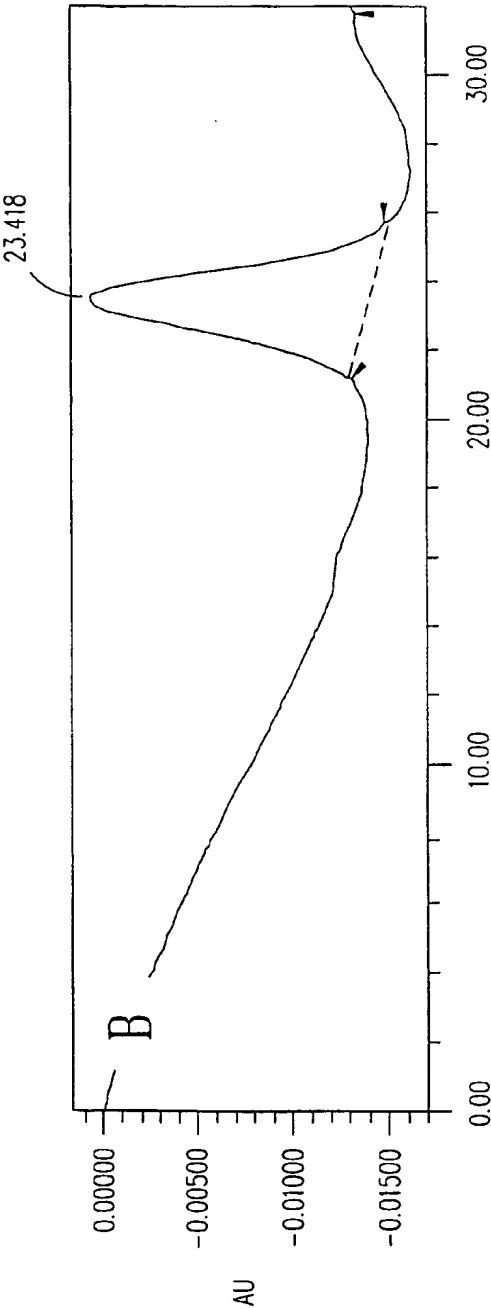


FIG. 12b

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/17535

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12P19/26 C08B37/00 A61K35/74 A61K31/73

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P A61K C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HELLERQVIST, C.G. ET AL.: "Studies on Group B beta-hemolytic Streptococcus. I. Isolation and partial characterization of an extracellular toxin" PEDIATRIC RESEARCH, vol. 15, no. 6, 1981, pages 892-898, XP002054823 cited in the application	17-28, 33-42
Y	---	1-16, 29-32
X	WO 91 04048 A (UNIV VANDERBILT) 4 April 1991 cited in the application see abstract see page 5, paragraph 3 see page 9, paragraph 4 - page 11, paragraph 1 ---	17-28, 33-46
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

10 February 1998

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category ²	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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